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Research article
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Effects of estradiol, progesterone and kisspeptin-1 concentrations at the time of insemination on conception rates in cattle

S. Kaya[1*], C. Kaçar[1], U.Ç. Arı[2], O. Merhan[3], M.C. Demir[1], T. Bekyürek[4], K. Gürbulak[4]

[1]Department of Obstetrics and Gynecology, Faculty of Veterinary, University of Kafkas, Kars, Turkey

[2]Department of Reproduction and Artificial Insemination, Faculty of Veterinary, University of Kafkas, Kars, Turkey

[3]Department of Biochemistry, Faculty of Veterinary, University of Kafkas, Kars, Turkey

[4]Department of Obstetrics and Gynecology, Faculty of Veterinary, University of Erciyes, Kayseri, Turkey

ABSTRACT: The present study aimed to investigate the effects of kisspeptin (Kiss-1), estradiol (E_2), and progesterone (P_4) concentrations at the time of artificial insemination (AI) on conception rates and whether or not there is a relationship between Kiss-1 and E_2 concentrations in cattle. Cows (n=100) raised on a semi-open free-stall barn and having corpus luteum were intramuscularly injected 25 mg of prostaglandin $F_{2α}$ (5 mg Dinoprost, Dinolytic®, Zoetis, Spain) and signs of estrus were checked for 5 days after injection. The cows that did not show heat for 5 days following the $PGF_{2α}$ injection were injected with the second dose of $PGF_{2α}$ on the 7th day and the oestrus was checked for half an hour, 3 times a day for 72 hours. Just prior to AI, pre-ovulatory follicle diameters were examined with transrectal ultrasonography and recorded. Then blood samples were collected. Blood serum samples were collected and stored until analyzed. Pregnancies were diagnosed with transrectal real time B-Mod ultrasonography on day 30±2 following AI. The mean follicular diameters at the time of AI were 17.82±0.01 mm. E_2, P_4, and Kiss-1 concentrations at the time of AI were determined in serum by ELISA. Mean concentrations of Kiss-1, estradiol and progesterone at the time of AI in all animals were 79.77±4.64 pg/mL, 117.09±1.48 pg/mL, and 0.61±0.02 ng/mL, respectively. The threshold value for kisspeptin, estradiol and progesterone concentrations were determined to be 82.03 pg/mL, 116.22 pg/mL and 0.93 ng/mL, respectively. Kiss-1 is a positive and significant predictor for the model but has limited accuracy. While Kiss-1, follicle diameter, milk yield, lactation period and AI number explains high accuracy (AUC=0.738) in pregnancy prediction, estrogen and progesterone are poor. İn addition, a significant correlation was found between serum kiss-1 concentration and follicle diameter, whereas no significant correlation was found between follicle diameter and serum estrogen or progesterone concentrations. In conclusion, Kiss-1 was found to be limited accurate in predicting pregnancy in cows, whereas estradiol and progesterone were not.

Keywords: *Cow, Estradiol, Follicular Diameter, Kiss-1, Progesterone, Pregnancy*

Corresponding Author:
S. Kaya, Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Kafkas University, 36300, Pasacayiri, Kars, Turkey.
E-mail address: emra-kafkas@hotmail.com

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INTRODUCTION

Kisspeptin (Kiss), encoded by the kisspeptin gene (Kiss-1), is a hypothalamic neuropeptide that affects gonadotropin-releasing hormone (GnRH) neurons by attaching to their receptors (GPR54) (Bhalakiya et al. 2019, Mishra et al. 2019, Okamura et al. 2013). This neuropeptide is known to play important roles in the neuroendocrine regulation of reproduction, puberty, and ovulation in mammals (Bhalakiya et al. 2019). Kisspeptin neurons are located on two different regions of the brain in many animal species, the caudal arcuate and rostral preoptic nucleus (Okamura et al. 2013). Estrogens create a positive feedback effect on Kiss neurons in the preoptic nucleus, increasing Kiss and also GnRH synthesis. Moreover, estrogens stimulate GnRH synthesis in pulse mode by a negative feedback on kisspeptin neurons in the arcuate nucleus (Okamura et al. 2013). Stimulated pulse frequencies of GnRH cause peak concentrations of LH which stimulates follicular maturation and ovulation (Okamura et al. 2013, Naniwa et al. 2013). Progesterone (P_4) and androgens in addition to estrogens cause negative feedback effects on Kiss neurons in the arcuate nucleus (Okamura et al. 2013). In a study carried out by Mondal et al. (2015), it was revealed that blood Kiss concentrations fluctuate depending on follicular development. It was also determined that blood Kiss concentrations significantly increased while antral follicles were changing to Graafian follicles.

In recent years, many studies have concentrated on Kisspeptin and its effect on reproduction; especially the effects of kisspeptin injections on reproductive parameters were investigated (Leonardi et al. 2018, Leonardi et al. 2020, Mondal et al. 2015). Kisspeptin injections have been found to increase plasma LH concentrations and follicle diameter and stimulate ovulation (Leonardi et al. 2018, Leonardi et al. 2020). Kisspeptins have also been reported to play a role in the development of cotyledon cell proliferation in the first trimester of pregnancy in cows (Martino et al. 2015). The present study aimed to determine whether or not any relationship exists between serum Kiss-1, estradiol (E_2), and P_4 concentrations at the time of AI and pregnancy rates.

MATERIALS AND METHODS

Animals

Holstein cows (n=100) fed on a total mixed ration (alfalfa silage, straw, soya pulp, grain corn silage, corn flakes) and kept in a semi-open free-stall barn on a private farm in Kayseri province were used in this study. Average milk yield during lactation was 33.5±0.9 liters 90-280 days after parturition and lactation numbers were 1-8. The number of inseminations in cows was between 1-8.

Study Methods

All animals were first checked by rectal palpation and ultrasonography (USG). Cows with corpus luteum were intramuscularly injected 25 mg of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (5mg/mL Dinoprost, Dinolytic®, Zoetis, Spain). Estrus detection was performed for half an hour, 3 times a day, during 5 days after $PGF_{2\alpha}$ injection. Cows showing estrus signs (standing to be mounted, restlessness, discharge of clear mucus, swelling of vulva) were separated to perform artificial inseminations (AI) 8-12 hours later. Cows not showing any estrus signs for 5 days following the $PGF_{2\alpha}$ injection were injected with the second dose of $PGF_{2\alpha}$ on the 7th day and were checked again for half an hour, 3 times a day, for 72 hours. Those with estrus signs after $PGF_{2\alpha}$ and dominant follicles were inseminated after transrectal ultrasonography examination. Just before AI, BCS were recorded. Diameter of dominant follicles was measured with the electronic calipers of the ultrasound (MINDRAY DP-20 Vet, Hasvet, Turkey) screen by taking the average of width and length from a frozen image (8 to 12 hours after estrous detection) just before AI. Blood samples from estrus cows were collected from the coccygeal vein, and then these cows were artificially inseminated. The blood samples were centrifuged at 3000 rpm for 10 minutes, and the obtained sera were stored at -20°C until the hormone analyses were conducted. Examination for pregnancy was performed on 30±2 days after AI by real time B-mode ultrasonography.

Cows were classified as primiparous and multiparous. Semen from a single bull was used for AI.

Kisspeptin, Estradiol, and Progesterone Analyses

Commercial kits were used to assess concentrations of E_2, P_4 (DRG Diagnostic GmbH®, ELISA kit, Germany), and Kiss-1 (Bovine Kiss-1, Sunlong Biotech®, ELISA, China). The assay sensitivity for Kiss-1 is 30 to 2000 pg/mL. Assay range of progesterone and estradiol are 0.14-40 ng/mL and 8.1-2400 pg/mL, respectively.

Statistical Analyses

Data analyses were conducted using IBM SPSS Statistics 26.0 (SPSS®, IL, USA) and Stata 12/MP4 statistical software. Data were initially summarized with descriptive statistics. Logistic regression analysis was used to examine the relationship between independent variables (follicular diameter, estradiol, kiss-1, progesterone, milk yield, AI number and lactation periods) and the log odds of the binary outcome variable (pregnant or non-pregnant). Predictor variables were tested prior to verify there was no violation of the assumption of the linearity of logit and multicollinearity. Each variable based on the results variables with a value of $P<0.25$, were investigated further and included in the model. Variables were excluded from the model by the backward elimination procedure. Maximum likelihood method was used for estimating the coefficients in the logistic regression model. Model's overall classification ratio was determined by using final model's estimated probabilities. The logistic procedure in SPSS was used to create Receiver Operating Characteristic curves (ROC) for pregnancy rate and predictor variables were evaluated with ROC analysis in order to determine critical thresholds for prediction of pregnancy rate. Scoring scale and interpretation of ROC curves was based on the area under curve (AUC) values and as follows: $AUC\leq 0.5$ = Poor, $0.5<AUC\leq 0.6$ = Accurate, $0.6<AUC\leq 0.7$ = Very Accurate, $0.7<AUC\leq 0.9$ = Highly Accurate, $0.9<AUC\leq 1.00$ = Excellent. A Spearman's correlation was run to assess the relationship between follicular diameters, Kiss-1, estradiol, progesterone concentrations and milk yield for all cows disregarding pregnancy and also for pregnant and non-pregnant cows. Results were analyzed and expressed as mean±standard error of means (x±SEM). The P value of <0.05 was considered to be statistically significant for all analysis.

RESULTS

Mean concentrations of Kiss-1, estradiol and progesterone at the time of AI in all animals were 79.77±4.64 pg/mL, 117.09±1.48 pg/mL, and 0.61±0.02 ng/mL, respectively. The mean follicle diameter was found to be 17.82 mm. It was determined that the pregnancy per AI was 56%. Serum Kiss-1, estradiol and progesterone concentrations at the time of AI in primiparous cows (n=35; 77.17±9.08 pg/mL, 114.96±2.47 pg/mL and 0.60±0.04 ng/mL, respectively) were similar to those in multiparous cows (n=65; 81.17±5.24 pg/mL, 118.23±1.85 pg/mL and 0.62±0.03 ng/mL, respectively).

A logistic regression analysis used to examine the relationship between independent variables (follicular diameter, estradiol, Kiss-1, progesterone, milk yield, AI number, lactation periods) and the log odds of the binary outcome variable (pregnant and non-pregnant). Follicular diameter, Kiss-1, milk yield, AI number and lactation period were found to contribute to the model; whereas estradiol and progesterone were found to be not significant to determine pregnancy in final model (Table 1). Regression analysis revealed a positive correlation between pregnancy rate and follicular diameter, or Kiss-1, or milk yield, and a negative one between pregnancy rate and AI number (coded as 0: ≤3, 1: >3) or lactation number (coded as 0: primiparous, 1: multiparous). Estradiol and progesterone are not significant predictor of pregnancy. Receiver operating characteristic curve for the logistic regression model presented in Figure 1. Kiss-1, follicle diameter, milk yield, lactation period and AI number explain high accuracy (AUC=0.738) in pregnancy prediction, whereas estrogen and progesterone a poor one. Classification matrix for logistic regression model is expressed in Table 2.

Table 1. Parameters of logistic regression model, based on pregnancy

	B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B)	
							Lower	Upper
Follicular Diameter (cm)	0.728	0.328	4.940	1	0.026	2.071	1.090	3.935
Estradiol	-0.005	0.016	0.112	1	0.738	0.995	0.964	1.026
Kiss-1	0.016	0.006	2.456	1	0.031	1.008	0.997	1.020
Progesterone	1.277	0.965	1.753	1	0.185	3.587	0.541	23.765
Milk Yield	0.050	0.029	3.094	1	0.049	1.052	0.994	1.113
AI Number	-0.910	0.476	3.653	1	0.036	0.402	0.158	1.023
Lactation number	-0.701	0.496	2.992	1	0.046	0.496	0.188	1.313
Constant	-15.957	6.492	6.042	1	0.014	<0.001		

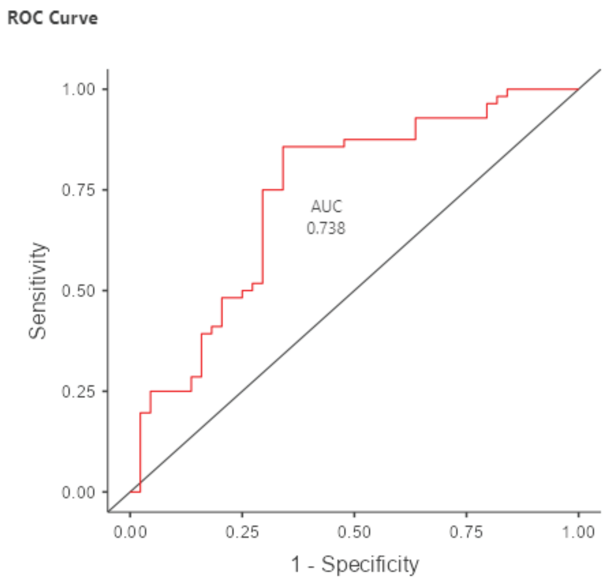

Figure 1. Receiver operating characteristic curve for the logistic regression model

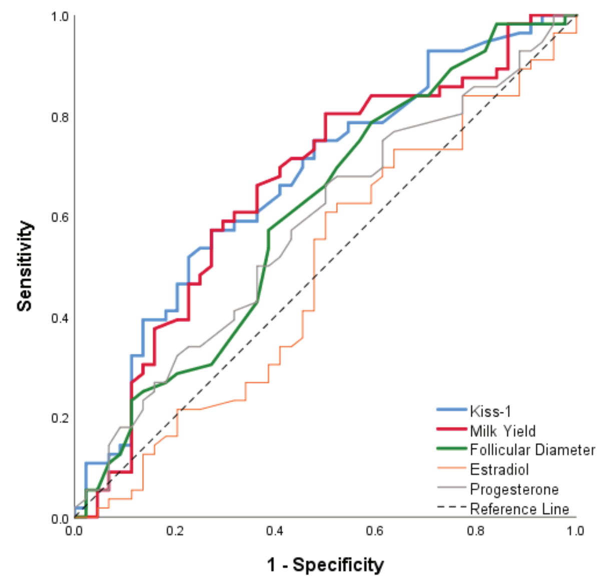

Figure 2. The ROC analysis curve for predictor variables

Table 2. Classification matrix for logistic regression model

Observed	Predicted		%Correct
	Non-Pregnant	Pregnant	
Non-Pregnant (n=44)	28	16	63.6
Pregnant (n=56)	13	43	76.8
Total			71

Table 3. Determination of critical threshold values for pregnancy prediction by ROC curve method

Predictor Variable	Critical Threshold	Area Under Curve	Sensivity	1-Specifity	p
Kiss-1 (pg/mL)	82.03	0.671	0.393	0.136	0.003
Milk Yield	33.35	0.659	0.571	0.273	0.006
Follicular Diameter (mm)	17.75	0.606	0.571	0.386	0.041
Estradiol (pg/mL)	116.22	0.388	0.625	0.523	0.372
Progesterone (ng/mL)	0.925	0.478	0.143	0.068	0.182

Table 4. The correlation matrix between follicle diameter, serum estradiol, Kiss-1 and progesterone concentrations

Variables	Follicle diameter	Estradiol	Kiss-1
spe-r	0.037	—	
p	0.715	—	
spe-r	0.266	-0.034	—
p	0.009	0.734	—
spe-r	0.06	-0.089	-0.013
p	0.551	0.381	0.897

Critical threshold, sensitivity, and specificity values for the Kiss-1, milk yield, follicular diameter, estradiol and progesterone concentrations determined as a result of the ROC analysis on cows are provided in Table 3. Only Kiss-1, milk yield and follicular diameter analyzed with ROC curves present a critical threshold value for pregnancy prediction. The ROC analysis curve is presented in Figure 2.

The results of the correlation analysis between follicle diameter, serum estradiol, Kiss-1 and progesterone concentrations are presented in Table 4., It was determined a weak correlation between only follicle diameter and Kiss-1 concentration (r=0.26, P=0.009).

DISCUSSION

Kiss expression in the hypothalamus via the Kiss-1 gene activates neurons producing GnRH and, in this way affects the regulation of follicle-stimulating hormone (FSH) and LH synthesis (Naniwa et al. 2013, Daniel et al. 2015, Gianetti and Seminara 2008, Hashizume et al. 2010). In a study carried out by Mondal et al. (2015), Kiss and LH levels during the estrus cycle were investigated in cow blood samples. It was determined that Kiss levels were the highest just one day before the pre-ovulatory LH peak. It has been reported that kisspeptin increases before preovulatory LH secretion due to its gonadotropin-releasing feature. Moreover, high Kiss concentrations during this period may be explained by high Kiss production of pre-ovulatory follicles. Plasma Kiss concentrations were determined as >3 ng/mL on the day of estrus. In a study by Rizzo et al. (2019), Kiss-10 concentrations in serum were determined to be between 114-149 pg/mL on the days 12-16 postpartum. In another study, Rizzo et al. (2018) collected blood samples from cows in estrus, and Kiss-10 concentrations (analyzed by ELISA assay) were 97.72±21.34 pg/mL. In the current study, the mean Kiss-1 concentration was 79.77±4.64 pg/mL for all cows just before AI (in estrus). Differences between study results for Kiss concentrations may originate from the different assay techniques, commercial kits used, and variants or variations of Kiss from different Kiss genes.

It has been determined that parenteral Kisspeptin injection has the ability to stimulate follicle development and/or ovulation by increasing FSH and LH levels in the studies conducted in recent years (Ahmed et al. 2009, Naniwa et al. 2013, Leonardi et al. 2018, Leonardi et al. 2020). In the current study, it was determined that the threshold value for Kisspeptin was 82.03 pg/mL. Our results could not be directly compared with other studies, as there was no study investigating whether the concentration of Kiss-1 affects pregnancy status. It was determined that the pregnancy rate was higher in those with high Kiss-1 concentrations. Nevertheless, it can be suggested that higher Kiss concentrations may increase the synthesis of LH in pregnant cows at the time of AI, and in this way, LH surge increases the ovulation rate (prevents anovulation or delayed ovulation) and an increased LH concentration may prevent the impotence of corpus luteum in pregnant cows.

Mondal et al. (2015a) found that the kisspeptin concentration was higher in animals with a larger follicle diameter (preovulatory follicle which is going to ovulate); moreover, kisspeptin concentration was the highest in estrus (1 day before preovulatory LH peak) and increased as the follicle diameter increased. Furthermore, the follicle diameter increased in animals injected with kisspeptin (Naniwa et al. 2013). It is reported that kisspeptin stimulates follicle development and this is achieved by stimulating GnRH/gonadotropin secretion (especially by inducing LH release) in cows (Naniwa et al. 2013). In the presented study, a weak correlation was found between follicle diameter and serum kiss-1 concentration. It has been reported that there is a positive correlation between follicle diameter on the day of insemination and pregnancy success in angus-crosses beef cows (Perry et al. 2005). In the present study, it was found that follicle diameter was insufficient to predict pregnancy rate (AUC=0.57, P=0.04), while Kiss-1 provided a more accurate estimation (AUC=0.67, P=0.003). In previous studies, it was determined that there is a positive correlation between ovulatory follicle diameter and serum estrogens concentration (Atkins et al. 2010, Sa´ Filho et al. 2009). However, no correlation was found in the presented study. In a study by Rizzo et al. (2019), a significant relationship was determined between serum estrogens concentrations and Kiss-10 concentrations on the 14th day postpartum. The reason for the concurrent peak in estrogens and Kisspeptin was reported to originate from the stimulation effect of Kiss-10 on the hypothalamus (GnRH) and hypophyses (gonadotropins), both of which cause follicular development. It was found that estrogen concentrations, increased with follicular development, increase Kiss-10 concentrations with a positive feedback mechanism. Similarly, in another study, it was determined that Kiss, estrogens, and P_4 concentrations in cows with cystic follicles are higher than those in healthy cows. It has been reported that a high-concentration of estrogens originating from cystic follicles leads to increased production of Kiss from the hypothalamic nucleus. Therefore, high Kiss concentrations cause tonic secretion of GnRH, which raises long-term high LH concentrations (Rizzo et al. 2018). Consequently, the LH surge needed for ovulation causes luteinization of follicles without ovulation which may lead to an increase in the P_4 concentration over the long term (Rizzo et al. 2018). In a study conducted in humans, a positive correlation was found between the kisspeptin concentration and the E_2 concentration in the follicular fluid, but there was no correlation between plasma kisspeptin concentration and serum estrogen concentration (Taniguchi et al. 2017). Similarly, in another study conducted in humans, no correlation was found between serum estrogen concentration and kisspeptin concentration (Latif and Rafique 2015). In the presented study, no correlation was found between serum estrogen and kisspeptin concentration, consistent with these studies. The reason (mechanism) of this is not fully known.

In conclusion, although there is a weak correlation between Kiss-1 and follicle diameter, no correlation was found between Kiss-1 concentration and serum estrogen and serum progesterone concentration. As far as we know, this is the first study investigating the Kiss-1 concentration in pregnancy prediction. Kiss-1 has limited accuracy in predicting pregnancy and it is obvious that it is not an alone marker. This study is a preliminary study. In future studies, it is recommended to compare the Kiss-1 threshold with different pregnancy-specific factors.

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